A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin

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The ability of *Listeria monocytogenes* to move within the cytosol of infected cells and their ability to infect adjacent cells is important in the development of infection foci leading to systemic disease. Interaction with the host cell microfilament system, particularly actin, appears to be the basis for propelling the bacteria through the host cell cytoplasm to generate the membraneous protrusions whereby cell-to-cell spread occurs. The *actA* locus of *L.monocytogenes* encodes a 90 kDa polypeptide that is a key component of bacterium – host cell microfilament interactions. Cloning of the *actA* gene allowed the identification of its gene product and permitted construction of an isogenic mutant strain defective in the production of the ActA polypeptide. Sequencing of the region encoding the *actA* gene revealed that it was located between the metalloprotease (*mpl*) and phosphatidyl-choline-specific phospholipase C (*plcB*) genes. Within the cytoplasm of the infected cells, the mutant strain grew as microcolonies, was unable to accumulate actin following escape from the phagocytic compartment and was incapable of infecting adjacent cells. It was also dramatically less virulent, demonstrating that the capacity to move intracellularly and spread intercellularly is a key determinant of *L.monocytogenes* virulence. Like all other virulence factors described for this microorganism, expression of the ActA polypeptide is controlled by the PrfA regulator protein. The primary sequence of this protein appeared to be unique with no extended homology to known protein sequences. However, an internal repeat sequence showed strong regional homology to a sequence from within the hinge region of the cytoskeletal protein vinculin.
Key words: actin nucleating protein/cell-to-cell spread/intracellular movement/*Listeria monocytogenes actA* gene/virulence gene

Introduction

Many bacterial species are capable of invading and multiplying in mammalian cells (Moulder, 1985). Currently available data suggest that these bacteria have evolved quite different strategies for entry into host cells, all involving some form of induced phagocytosis (Finlay and Falkow, 1989; Falkow, 1991). Once within the infected cell, two distinct modes of intracellular survival for such microorganisms have been distinguished. Some invasive microorganisms like *Salmonella* or *Yersinia* species remain and multiply within a membrane bound phagocytic vacuole (Moulder, 1985; Finlay and Falkow, 1989). Intercellular spread of such bacteria may follow routes utilized by intracellular organelles by transcytosing from the apical to the basal pole of these cells as has been suggested for example for *Salmonella cholerasuis* (Finlay and Falkow, 1988). Other invasive microorganisms like *Shigella* spp. and *Listeria monocytogenes* induce rapid lysis of the phagocytic vacuole and interact subsequently with the host cell actin-containing microfilament system to effect intracellular movement and cell-to-cell spread (Clerc and Sansonetti, 1987; Bernadini *et al.*, 1989; Tilney and Portnoy, 1989; Mounier *et al.*, 1990; Sansonetti, 1991). Apart from the involvement of actin, the mechanical basis of bacterial movement is not clear. Indeed for *L.monocytogenes* it is not known whether this process is controlled by bacterial products.

Previous attempts to identify bacterial gene products involved in the nucleation of actin filaments and cell-to-cell spread in *L.monocytogenes* were unsuccessful (Sun *et al.*, 1990). Further studies, however, indicated that the PrfA regulator protein which regulates the expression of listerial virulence gene products such as listeriolysin (Hly), phosphatidylinositol-specific phospholipase C (PlcA), metalloprotease (Mpl) and phosphatidylcholine-specific phospholipase C (PlcB) also controls actin-mediated intracellular movement and intercellular spread (Mengaud *et al.*, 1991b; Chakraborty *et al.*, 1992). We and others have initiated studies to mutate *prfA*-inducible genes sequentially and investigate their roles at different stages following bacterial infection.

In this communication we report on the identification of a 90 kDa polypeptide elaborated by *L.monocytogenes* that induces actin nucleation around bacteria following escape from the phagocytic compartment. Bacteria mutant for the gene designated *actA* grow intracellularly as microcolonies and are unable to accumulate actin or to infect adjacent cells. Such mutants are dramatically less virulent, demonstrating that the capacity to spread intracellularly and to infect adjacent cells is a key determinant of *L.monocytogenes* virulence.

Results

Cloning of the actA gene of Listeria monocytogenes

The DNA region following the *mpl* gene of *L.monocytogenes* was found on a 2.9 kb *Ssp*I fragment following Southern hybridization of chromosomal DNA using an oligonucleotide probe derived from a region located 3′ to the *mpl* gene on
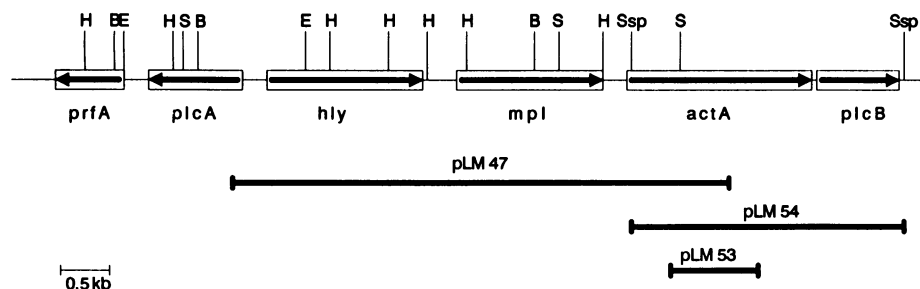
Fig. 1. Partial restriction map of the chromosomal region harbouring the prfA, plcA, hly, mpl, actA and plcB genes of L.monocytogenes EGD. The locations of these genes are shown by open boxes together with the direction of transcription of the genes. Thick black lines represent various lengths of L.monocytogenes DNA inserted into the pUC18 vector. Restriction endonuclease sites are as follows: B, BamHI; E, EcoRI; H, HindIII; S, SphI; and Ssp, SspI.

pLM47. This fragment was cloned into the SmaI site of the vector plasmid pUC18 to give pLM54 (Figure 1). The nucleotide sequence of the entire insert was determined and the combined sequence derived from the end of the mpl gene on pLM47 and pLM54 is presented in Figure 2A.

Analysis of the nucleotide sequence revealed the presence of two further open reading frames (ORFs) following the mpl gene (Domann et al., 1991; Mengaud et al., 1991a). The first reading frame comprised 1917 nucleotides (nt 202−2118) and was located 197 nt 3' to the TGA stop codon of the metalloprotease gene. This reading frame encoded a 639 amino acid polypeptide with a predicted mol. wt of 70 419 and had a calculated pI of 4.77. It was preceded by a consensus ribosome binding site sequence typical for listerial genes located 8 nt upstream of its initiation codon GTG. A palindromic sequence, 5'-TTAACAAATGTTAG-3' (Mengaud et al., 1989; Leimeister-Wächter et al., 1990), thought to be responsible for PrfA-mediated regulation of virulence genes in L.monocytogenes was present 183 nt upstream of the GTG initiator codon of this putative polypeptide. The deduced polypeptide was rich in secondary structure, contained an unusually high amount of proline (9%) and the sequence Arg−Gly−Asp starting at residue 360. The central region of the molecule is made up largely of repeated sequences, the longest of which comprises 34 amino acid residues (Figure 2B).

A search of signal peptide sequences based on the predictions of the (−3,−1) rule of von Heijne (1986) indicated no N-terminal signal sequences but revealed the presence of two regions internal to the polypeptide having all features of signal peptide sequences and with cleavage sites following the amino acid residues at positions 293 and 328. The C-terminus harboured a 20 amino acid transmembrane helix followed by a six amino acid charged tail and appeared to be anchored to the bacterial cell wall. The gene encoding the polypeptide has been designated actA (see below).

The putative start codon of the second ORF is located 36 nt downstream of the first ORF and was 867 nt long. The predicted reading frame encoded a protein of 289 amino acids with a calculated mol. wt of 33 193 and a predicted pI of 8.34. The proposed ATG start site of the second ORF is preceded by a ribosome binding site (GGAG at bp −8), and the predicted sequence contains 25 N-terminal amino acid residues consistent with established signal sequences found in transported prokaryotic proteins (von Heijne, 1986). Computer searches for homologous amino acids in the GenBank and EMBL databases revealed homologies between

this reading frame and the phosphatidylcholine-specific phospholipase C of Bacillus cereus (Gilmore et al., 1989) and the alpha toxin of Clostridium perfringens which also has a phospholipase exhibiting preferential activity for phosphatidylcholine (Johansen et al., 1988; Titball et al., 1991). Recently, a 29 kDa polypeptide exhibiting lecithinase activity has been purified from L.monocytogenes (Geoffroy et al., 1991). Like the B.cereus enzyme, this phospholipase is also secreted as a preproenzyme with the N-terminal sequence of the mature enzyme predicted to start at amino acid residue 53 (Johansen et al., 1988). The gene encoding this activity has been designated plcB (Figure 2A; see also Vasquez-Boland et al., 1992).

Construction of the actA insertion mutant

In order to examine the role of the actA gene in L.monocytogenes infections, a site-directed insertion mutant was constructed by chromosomal integration of a suicide vector containing a portion of the actA gene into the chromosomal copy of the gene by homologous recombination as described previously (Chakraborty et al., 1992; Figure 3A). This was shown to be the case by Southern blot hybridization of a representative L.monocytogenes strain transformed with plasmid pAUL53. Genomic DNA from either the parental L.monocytogenes strain EGD or its derivative harbouring integrated pAUL53 were cleaved with the restriction endonucleases SphI and BamHI (Figure 3B). A radiolabelled probe corresponding to the entire length of the actA gene was used to detect integration events. Two SphI fragments of 3.7 and 1.3 kb hybridized strongly to the actA probe in the wild type parental strain. In the mutant strain two additional SphI fragments of 2.2 and 0.7 kb respectively hybridized and were also detected with the radiolabelled probe. Similarly, two new BamHI fragments of 24.8 and 2.2 kb hybridized to the probe in the putative actA mutant while a single BamHI fragment of 17.0 kb was seen with the parental strain (Figure 3B). The insertion creates two half copies of the actA gene, but only the promoter-proximal half copy of this gene would encode for a truncated version encoding the first 425 amino acids of the ActA polypeptide (Figure 3A; see also Figure 4, lane 3).

Detection of the ActA polypeptide

To confirm that the L.monocytogenes actA mutant was indeed defective for the production of the intact ActA polypeptide, we needed a means of detecting its expression.

To do this, antisera were produced in rabbits against three synthetic polypeptides corresponding to amino acid residues from various regions of the ActA polypeptide. Immunoblotting experiments performed with these antisera showed, unexpectedly, specific immunological cross-reactivity to a 90 kDa polypeptide in the parental strain and a 63 kDa polypeptide in the *act*A1 mutant (Figure 4a and b; lanes 2 and 3). The polypeptide recognized by the antisera is larger than the predicted size of 70 kDa deduced from the primary sequence. However, secondary structural features of the polypeptide may have led to its slower migration in SDS−polyacrylamide gels. Likewise, cross-reactivity to a 63 kDa polypeptide was detected in the mutant strain although a truncated polypeptide of 47 kDa was predicted from the primary sequence. The ActA polypeptide was present in the cell wall fraction as well as in supernatant fluids of the wild type strain (our unpublished results). In the case of the mutant strain the truncated polypeptide was only detected in supernatant fluids. Since no signal peptide sequence could be detected from the primary sequence, the amino acid residues at the N-terminal end of the secreted ActA and ActA1 polypeptides were examined. For each polypeptide the first 30 amino acids were determined by microsequencing. The N-terminal amino acid residues were identical for both polypeptides and corresponded to the alanine residue at position 30 of the ActA sequence. Hence, the mature form of the ActA polypeptide is a secreted protein comprising 610 amino acids.

Expression of the ActA polypeptide is regulated by PrfA

The presence of a palindromic sequence similar to that found preceding the promoters of other PrfA-regulated genes suggested that the expression of the ActA polypeptide might also be regulated by PrfA. To examine this, primer extension analysis was performed with total RNA isolated from the wild type parental strain and its isogenic mutant defective in the *prf*A gene (Chakraborty *et al.*, 1992). No transcriptional start sites could be determined from the RNA derived from the *prf*A1 mutant while a weak, albeit clear, start site was detected in the parental strain (Vasquez-Boland *et al.*, 1992; our unpublished results). The transcriptional start site is located at nucleotide position 52, 33 nt away from the end of the consensus palindrome sequence (Figure 2A). To obtain further evidence that expression of the ActA polypeptide is regulated by PrfA, immunoblotting experiments were also performed with culture supernatants derived from the wild type and *prf*A1 strains using specific anti-ActA antisera. The ActA polypeptide was only detectable in strains harbouring an intact *prf*A gene. Hence, expression of the *act*A gene, like that of other listerial virulence genes, is regulated by PrfA (Figure 4a and b; lanes 2 and 4).

Invasive and intracellular properties of the actA1 mutant

The ability of the parental strain and the mutant to form plaques in L929 fibroblasts was examined. An isogenic mutant defective for the *prf*A gene (*prf*A1) was included as a control (Chakraborty *et al.*, 1992). The results of these experiments are summmmarized in Table I and indicate that, like the *prf*A1 mutant, the *act*A1 mutant was unable to form plaques on these monolayers. We next examined the ability

of the mutant to survive and multiply intracellularly. The *act*A1 mutant was internalized as readily as the wild type parental strain while the *prf*A1 mutant showed 5- to 10-fold less internalized bacteria than either of the two other strains. Quantitative plate counts of numbers of bacteria in the cytoplasm of L929 cells showed that the *act*A1 mutant grew as proficiently as (or maybe even slightly better than) its parental strain while the *prf*A1 mutant was clearly also affected in its ability to grow intracellularly (Table I). We therefore concluded that the inability of the *act*A1 mutant to form plaques on L929 monolayers was not due to an impairment in its invasive phenotype and could not be attributed to its lack of replication within the infected host cell.

The cell biological defect in this mutant was characterized by studying preparations of Henle cells infected with either the parental or isogenic mutant by double fluorescent labelling using rhodamine-labelled phalloidin to stain F-actin and indirect immunofluorescence to stain the bacteria. As expected, phalloidin staining of the parental strain showed that the bacteria were brightly fluorescent with some of the bacteria followed by a bright fluorescent trail (Figure 5, a−d). In the case of the mutant we were unable to detect any actin surrounding the intracellular bacteria (Figure 5e and f). This mutant had also lost its capacity to spread intracellularly and to infect adjacent cells. *L.monocytogenes* *act*A1 was localized within a cytoplasmic area close to the nucleus and grew locally forming a microcolony (Figure 5e and f).

Since it was impossible to distinguish by light microscopy whether the microcolony formed was enveloped by a surrounding phagocytic membrane or whether the bacteria were growing freely in the cytoplasm, we used electron microscopy to examine Henle cells infected with the mutant strain. This method also allows direct visualization of the actin filament meshwork surrounding infecting bacteria. These data revealed that the mutant bacteria were present in the host cytoplasm and remained sequestered in close proximity to the nucleus unlike the parental strain which was dispersed throughout the cell. In addition, unlike the parental strain (Figure 6a and c), mutant bacteria were totally devoid of the dense layer of microfilaments that surrounded the wild type parental bacteria (Figure 6b and d). This was in perfect agreement with the phalloidin staining results, indicating that the mutant strain was indeed unable to accumulate actin.

The actA gene is a virulence gene of L.monocytogenes

The effect of the *act*A1 mutation on the virulence of *L.monocytogenes* was assessed by its ability to grow in host tissues when injected intravenously into mice. Unlike its parent strain which showed significant growth of bacteria in the spleens of infected mice, the isogenic *act*A1 mutant strain was rapidly eliminated from host tissues within two days following infection and showed no further growth (Table II). Hence, the capacity of virulent *L.monocytogenes* to move intracellularly and to infect adjacent cells is a key determinant of virulence for this microorganism.

Discussion

Cell biological studies of *L.monocytogenes* following infection of tissue culture cells has led to a fairly detailed morphological description of events following invasion

A

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                    +++++++++++++                                      *
        TGATTAACAAATGTTAGAGAAAAATTAATTCTCCAAGTGATATTCTTAAAATAATTCATGAATATTTTTTCT  72
                    -35                        -10      +1


        TATATTAGCTAATTAAGAAGATAACTAACTGCTAATCCAATTTTTAACGGAACAAATTAGTGAAAATGAAGG  144

                                            RBS          **************
        CCGAATTTTCCTTGTTCTAAAAAGGTTGTATTAGCGTATCACGAGGAGGGAGTATAAGTGGGATTAAACAGA  216
                                                          VALGlyLeuAsnArg    5

        TTTATGCGTGCGATGATGGTGGTTTTCATTACTGCCAATTGCATTACGATTAACCCCGACATAATATTTGCA  288
        PheMETArgAlaMETMETValValPheIleThrAlaAsnCysIleThrIleAsnProAspIleIlePheAla   29

        GCGACAGATAGCGAAGATTCTAGTCTAAAACACAGATGAATGGGAAGAAGAAAAAACAGAAGAGCAACCAAGC  360
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        ProAlaIleGlnValGluArgArgHisProGlyLeuProSerAspSerAlaAlaGluIleLysLysArgArg  149

        AAAGCCATAGCATCATCGGATAGTGAGCTTGAAAGCCTTACTTATCCGGATAAACCAACAAAAGTAAATAAG  720
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        LysLysValAlaLysGluSerValAlaAspAlaSerGluSerAspLeuAspSerSerMETGlnSerAlaAsp  197

        GAGTCTTCACCACAACCTTTAAAAGCAAACCAACAACCATTTTTCCCTAAAGTATTTAAAAAAAATAAAAGAT  864
        GluSerSerProGlnProLeuLysAlaAsnGlnGlnProPhePheProLysValPheLysLysIleLysAsp  221

        GCGGGGAAATGGGTACGTGATAAAATCGACGAAAATCCTGAAGTAAAGAAAGCGATTGTTGATAAAAGTGCA  936
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        GAACCGAGCTCATTCGAATTTCCACCACCACCTACGGATGAAGAGTTAAGACTTGCTTTGCCAGAGACGCCA  1152
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        ATGCTTCTTGGTTTTAATGCTCCTGCTACATCGGAACCGAGCTCGTTCGAATTTCCACCGCCTCCAACAGAA  1224
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        AATGTGTTTAATAAAAAAACTACAACGAAAACAGTGACTAAAAAACCAACCCCTGTAAAGACCGCACCCAAAG  1728
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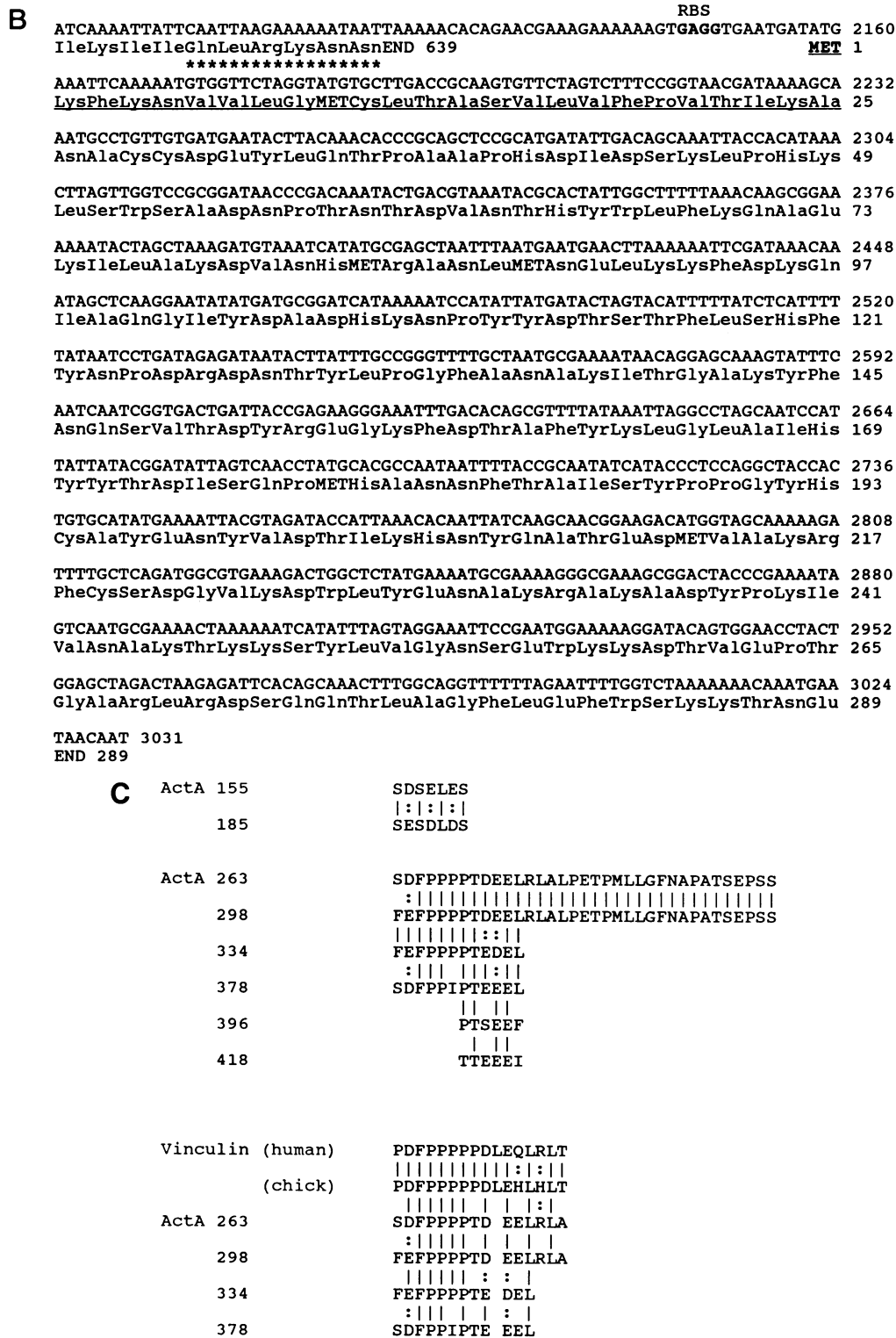
RBS

B

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       ****

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<u>LysPheLysAsnValValLeuGlyMETCysLeuThrAlaSerValLeuValPheProValThrIleLysAla</u> 25

AATGCCTGTTGTGATGAATACTTACAAACACCCGCAGCTCCGCATGATATTGACAGCAAATTACCACATAAA 2304
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GGAGCTAGACTAAGAGATTCACAGCAAACTTTGGCAGGTTTTTTAGAATTTTGGTCTAAAAAAACAAATGAA 3024
GlyAlaArgLeuArgAspSerGlnGlnThrLeuAlaGlyPheLeuGluPheTrpSerLysLysThrAsnGlu 289

TAACAAT 3031
END 289

C   ActA 155   SDSELES
                            |:|:|:|
            185   SESDLDS

  ActA 263   SDFPPPPTDEELRLALPETPMLLGFNAPATSEPSS
                            :|||||||||||||||||||||||||||||||||||
            298   FEFPPPPTDEELRLALPETPMLLGFNAPATSEPSS
                            ||||||||::||
            334   FEFPPPPTEDEL
                            :||| |||:||
            378   SDFPPIPTEEEL
                            || ||
            396   PTSEEF
                            | ||
            418   TTEEEI

  Vinculin (human)   PDFPPPPPDLEQLRLT
                            ||||||||||||:|:||
           (chick)   PDFPPPPPDLEHLHLT
                            |||||| | | |:|
  ActA 263   SDFPPPPTD EELRLA
                            :|||||| | | | |
            298   FEFPPPPTD EELRLA
                            |||||| : : |
            334   FEFPPPPTE DEL
                            :||| | | : |
            378   SDFPPIPTE EEL

Fig. 2. **A**. The nucleotide sequences and deduced amino acid sequences of the *act*A and *plc*B genes. The palindromic sequence thought to be responsible for PrfA-mediated regulation of virulence genes in *L.monocytogenes* is indicated by a row of plus signs and the oligonucleotide used to determine the transcription start site of the *act*A gene is underlined. The promoter region and the transcriptional start site are indicated as −35, −10 and +1 respectively. The ribosome binding sites (RBS) and the signal peptide sequences of the *act*A and *plc*B genes are indicated by continuous lines. The oligonucleotides used to amplify the *act*A gene for the Southern hybridization experiments are indicated by a row of asterisks. **B**. Alignment of internal repeat sequences of the *act*A gene product and homology to the proline-rich region of vinculin. The repeats were aligned visually. Identities and conservative changes are denoted by the colon and period, respectively.

(Tilney and Portnoy, 1989; Mounier *et al.*, 1990). These studies have revealed that intracellular movement of *L.monocytogenes* within the cellular cytoplasm of these cells is independent of organelle, i.e. microtubule dependent, movement, and requires other components of the cell cytoskeleton particularly actin. In this study we have
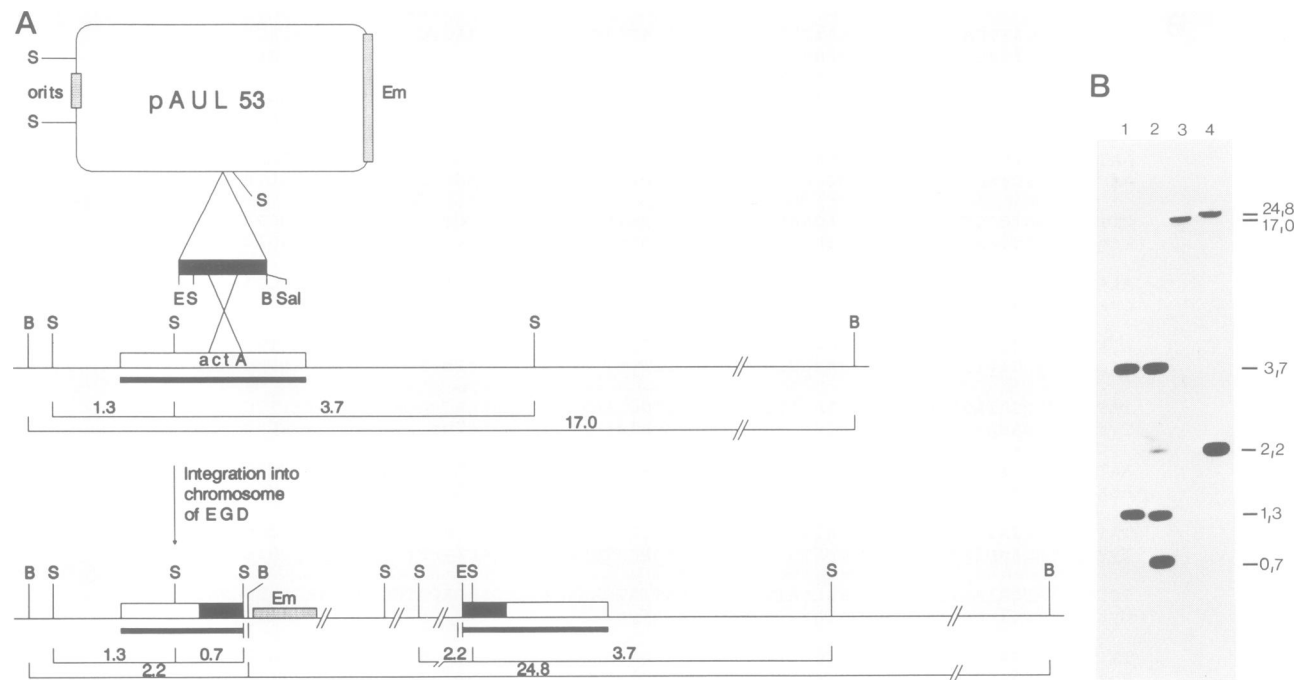
Fig. 3. A. Southern blot analysis of pAUL53 integration events. The thick black line represents the internal region from the *act*A region bracketed within a *Eco*RI–*Sal*I fragment. These restriction sites derived from the pUC18 cloning vector. Homologous recombination (×) integrates pAUL53 into the chromosome to give fragments indicated in the diagram. B = *Bam*HI, E = *Eco*RI, S = *Sph*I and Sal = *Sal*I restriction sites. **B.** Chromosomal DNA samples from the strains indicated below were digested with either *Sph*I or *Bam*HI, electrophoresed in an agarose gel, transferred to nitrocellulose and hybridized to a ^{32}P-labelled *act*A probe. The molecular weights (in kb) of bands appearing on the autoradiograph are also indicated in the diagram in panel A. Lanes 1 and 3 contain EGD wild type strain and lanes 2 and 4 contain DNA from the *act*A1 mutant digested with *Sph*I and *Bam*HI respectively.

characterized a *L.monocytogenes* gene that is required for accumulation of actin around the bacterium following escape from the phagocytic compartment. A mutation within this gene renders the infecting bacteria incapable of movement within the cytoplasm and unable to spread intercellularly. In addition, this mutant is avirulent when tested in the mouse infection model.

The ActA polypeptide was found experimentally to encode a 29 amino acid signal peptide, indicating that it is an exported protein. An intriguing observation is that there are two further signal peptide sequences with cleavage sites that correspond exactly to the ends of two 34 amino acid repeats within the central region of the molecule. The presence of a C-terminal transmembrane α-helical region followed by a positively charged tail suggests that this protein is anchored to the cell membrane. Evidence for the presence of such an anchor region at its C-terminal end comes from the detection of the truncated ActA1 polypeptide, which lacks the C-terminal 214 amino acids and is only found in the supernatant fluid of the mutant. A sequence motif, KxxxLPATKP, typical of surface proteins of Gram-positive cocci, was found starting at amino acid residue 509 of the pre-ActA polypeptide. Such sequences have often been found in close proximity to a hydrophobic amino acid segment located at the very C-terminal end of such molecules (Fischetti *et al.*, 1990). This was not the case for the motif in the ActA polypeptide, so its function at this time is not clear.

Expression of the ActA polypeptide in *L.monocytogenes* is, like all known virulence factors for this species, controlled by the PrfA regulator. Here we have shown that the consensus palindromic sequence that is present in the −40 region of all PrfA-regulated genes is also present in a similar
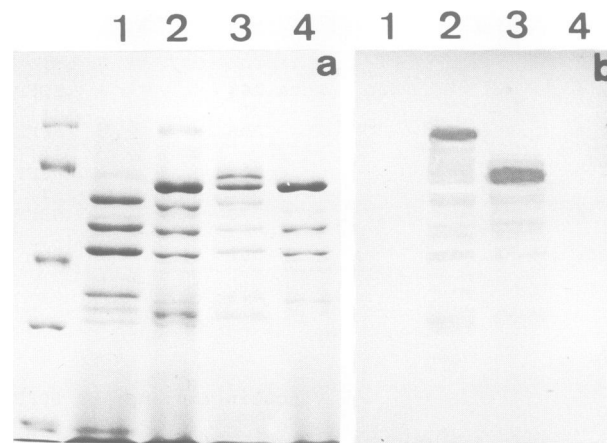

Fig. 4. Immunological detection of the ActA polypeptide in culture supernatants derived from *Listeria innocua* (lane 1), *L.monocytogenes* EGD (lane 2), *L.monocytogenes act*A1 (lane 3) and *L.monocytogenes prf*A1 (lane 4). **(A)** Coomassie-stained gel; **(B)** corresponding immunoblot, reacted with an antiserum raised against the peptide comprising amino acid residues 41–54. Molecular mass standards at the left are as follows: 92, 68, 46, 29 and 14 kDa (top to bottom).

spatial region of the *act*A promoter (Mengaud *et al.*, 1989; Domann *et al.*, 1991; Figure 2A). Northern blot analysis using DNA probes from within the *act*A and *plc*B genes reveal the presence of two large transcripts of 5400 and 3500 nt respectively, while a DNA probe consisting of the *mpl* gene detects the 5400 nt transcript in addition to its own transcript of 1800 nt (Leimeister-Wächter *et al.*, 1991; Mengaud *et al.*, 1991b; Chakraborty *et al.*, 1992; our

Table I. Uptake and replication of *Listeria monocytogenes* EGD strains in L929 cells

EGD strain	Inoculum	T_0 (cell associated) (% Inoculum)	T=1 h (% associated)	T=4 h	T=7 h	Generation time (min)	Plaque formation
pERL-3	4.5×10^5	$2.4 \times 10^5 \pm 1.6 \times 10^{4*}$ (53%)	$1.2 \times 10^4 \pm 8 \times 10^2$ (4.8%)	$6.8 \times 10^4 \pm 3.3 \times 10^4$	$6.3 \times 10^5 \pm 2.1 \times 10^4$	63	+
*prf*A1	6.1×10^5	$1.9 \times 10 \pm 4.5 \times 10^3$ (30%)	$1.7 \times 10^3 \pm 200$ (0.9%)	$2.2 \times 10^3 \pm 100$	$5.2 \times 10^3 \pm 1500$	223	−
*act*A1	4.9×10^5	$2.2 \times 10^5 \pm 3 \times 10^4$ (45%)	$2.2 \times 10^4 \pm 350$ (10.2%)	$2.6 \times 10^5 \pm 9 \times 10^3$	$1.7 \times 10^6 \pm 1.6 \times 10^5$	57	−

* c.f.u. per monolayer ($n=3$)
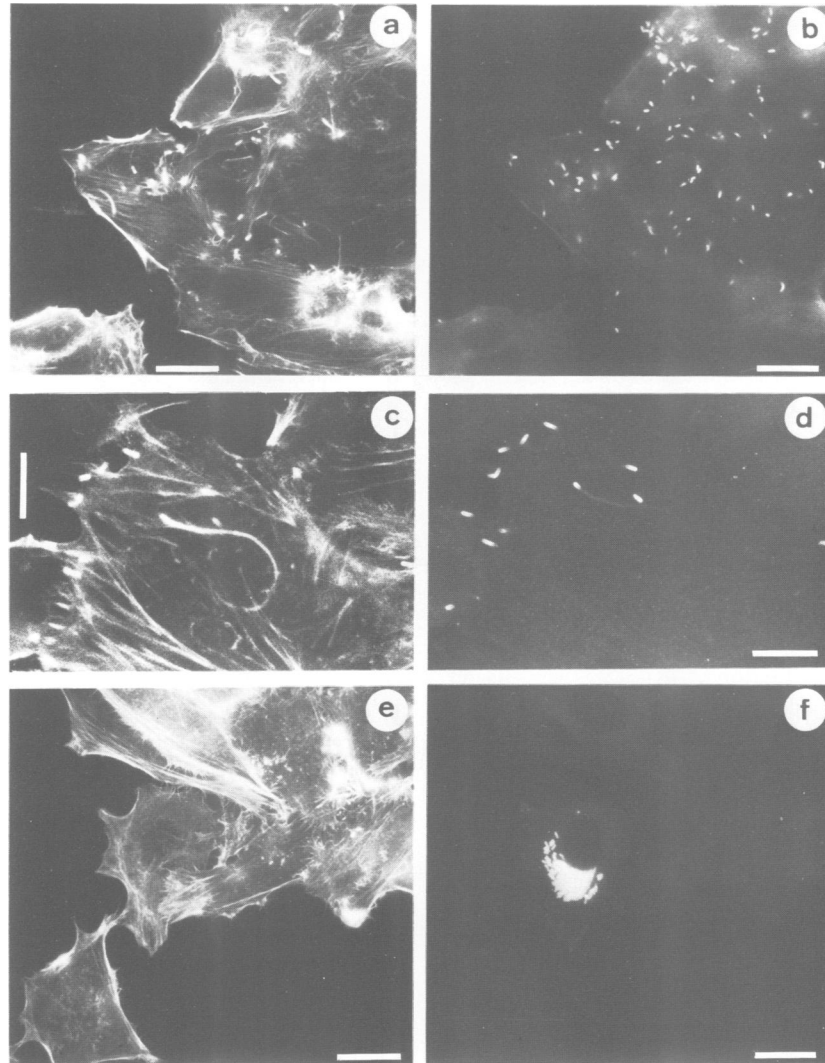
Fig. 5. Fluorescence microscopy of Henle cells (Intestine 407) infected with *L.monocytogenes* EGD (a–d) or its isogenic *act*A1 mutant (e and f). Five hours after infection, cells were processed for double fluorescence microscopy using rhodamine-labelled phalloidin (a, c and e) and a rabbit antiserum to formalinized *L.monocytogenes* EGD followed by fluorescein-labelled secondary antibodies (b, d and f). The overview (panels a and b) shows that in cells infected with the parental strain EGD, extensive bacterial spreading has occurred. Actin (as detected by phalloidin staining) has accumulated around the intracellular bacteria, occasionally giving rise to typical 'comets'. A higher magnification of the periphery of an infected cell (c and d) shows several bacteria surrounded by actin and a single bacterium followed by a trail of actin that is typical of the 'comet' structure. The length of the actin tail is dependent on the time that a bacterium has spent in the host cell cytoplasm. In cells infected with the *act*A1 mutant, no actin is observed around individual bacteria (compare a, c and e). These bacteria grow locally as a microcolony in the perinuclear area and are unable to infect adjacent cells. Bars represent 20 μm (a, b, e and f) and 40 μm (c and d).

unpublished results). This suggests that the *act*A and *plc*B genes are part of two operons with transcription sites originating from the *mpl* and *act*A promoters respectively. The recent cloning and sequencing of a 5600 bp region downstream of the *mpl* gene of a serotype 1/2c *L.monocytogenes* strain indicates an identical genome organization of genes in this region and provides strong evidence for the presence of an operon structure of genes
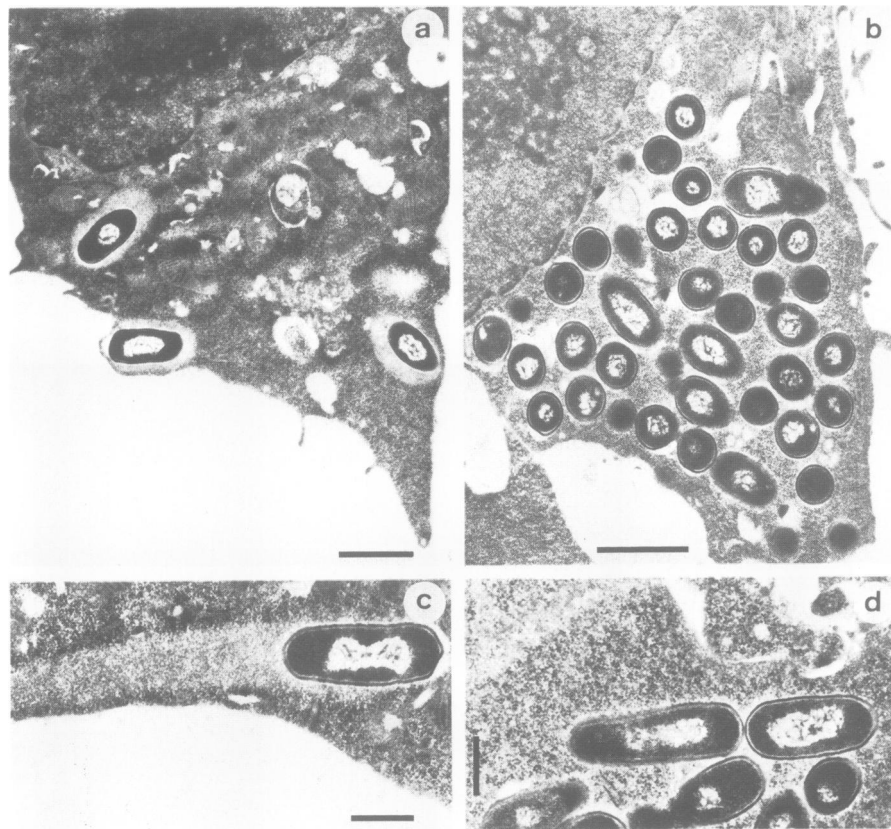
Fig. 6. Transmission electron micrographs of ultrathin sections of *L.monocytogenes* EGD (a and c) and the isogenic *act*A1 mutant (b and d) 5 h following infection of Henle cells. Panels **a** and **c** show Henle cells infected with the wild type EGD strain. Several bacteria surrounded by dense spongy material are seen close to the periphery of the infected cell in panel a. In panel c, a single bacterium with a typical trail of actin is shown. Panels **b** and **d** show infection with the *act*A1 mutant. A cluster of bacteria is growing locally adjacent to the host nucleus (b). Neither the dense spongy material nor a membrane is visible around the mutant bacteria (b and d). Bars represent 1 μm (a and b) and 0.5 μm (c and d).

located downstream of the listeriolysin gene (Vasquez-Boland *et al.*, 1992).

The *act*A1 mutant strain was not impaired for uptake, escape from the phagocytic compartment or intracellular replication. Although this suggests that the Arg−Gly−Asp sequence motif is not required for adhesion of these strains to the eukaryotic cell surface (Roushlahti, 1991), further studies with site-directed mutations within this region or competition of adhesion with RGD-containing peptides will be required to resolve this issue clearly. The somewhat faster replication of the *act*A1 mutant strain in the host cytoplasm than its wild type counterpart is reminiscent of *Shigella flexneri* mutants that are invasive but Sereny-negative (Lett *et al.*, 1989; Pal *et al.*, 1989), and suggest that the apparent differences in bacterial multiplication are a result of localized growth in the case of the mutant compared with spreading and multiplication of the wild type strain.

The growth of the mutant strain as microcolonies and its inability to accumulate actin and form plaques in monolayers of L929 cells suggests that the ActA polypeptide is essential for both intracellular movement and intercellular spread. Site-directed insertion mutants within the *mpl* and *plc*B genes indicate that both types of mutant are still capable of accumulating actin following escape from the phagocytic compartment (Mengaud *et al.*, 1991a; our unpublished results). Indeed, it has recently been shown that a *plc*B mutant is defective for escape from the double membrane compartment following intercellular spread (Vasquez-Boland *et al.*, 1992).

Table II. Growth of *Listeria monocytogenes* strains in spleens of mice following intravenous injection

Strain	Log$_{10}$ numbers injected i.v.	Log$_{10}$ numbers of bacteria/g of spleen[a]	
		Day 2	Day 4
EGD	3.3	6.2	>7.0
EGD *act*A1	4.9	1.6	−

[a] Log$_{10}$ mean value of three mice

Even though the identification of an actin-nucleating protein produced by *L.monocytogenes* provides a basis for envisaging how actin might accumulate around the infecting bacterium, several important questions remain to be addressed. Does the ActA polypeptide interact directly with actin? What is the basis of polarization and movement of the bacteria within the host cytoplasm? The GenBank (version 68) and SwissProt (version 21) databases contain no polypeptides with significant similarity to ActA. Direct comparison of the ActA sequence with that of the 120 kDa VirG (IcsA) polypeptide of *Shigella flexneri*, a protein that is required for the interaction of these bacteria with host cell microfilaments (Bernadini *et al.* 1989; Lett *et al.*, 1989), indicated that these polypeptides are not homologous. Hence, although these bacteria have strikingly similar intracellular behaviour, and although the phenotypes of *vir*G (*ics*A)

mutants of *S.flexneri* are similar to those of the *act*A1 mutant described here, the genes required for interaction with host cell microfilaments appear to have evolved independently in these bacteria.

When the same databases were searched with the repeat II sequence motif (Figure 2B), strong regional homology was detected to the proline-rich hinge region of the cytoskeletal protein vinculin (Weller *et al.*, 1990). This region separates the globular head domain of vinculin from its C-terminal tail but no function has yet been assigned to this sequence. Vinculin is associated with a family of proteins specialized in cell—cell and cell—substrate contacts where it is thought to form a complex with other cytoskeletal components to anchor microfilaments to the membrane (Burridge *et al.*, 1990).

The *act*A gene is part of a cluster of virulence genes in *L.monocytogenes* whose expression is controlled by the PrfA regulator (Mengaud *et al.*, 1991b; Chakraborty *et al.*, 1992; Vasquez-Boland *et al.*, 1992). This suggests that these bacteria actively orchestrate their movement in the eukaryotic host cell. Hence further PrfA-regulated gene products may be involved in polymerization and polarization of the host cell microfilaments required for propelling bacteria through the cytoplasm and to generate the protrusions by which adjacent cells are infected. Identification of the genes involved in these processes and studies on their interaction with host cell proteins will enable us to unravel the molecular mechanisms that underly this fascinating aspect of the microbial cell—host interaction.

Materials and methods

Bacterial strains, media and reagents

The weakly haemolytic *L.monocytogenes* serotype 1/2a EGD strain pERL-3, its isogenic *prf*A1 derivative and *L.innocua* have been described previously (Leimeister-Wächter, 1989; Chakraborty *et al.*, 1992). The *Escherichia coli* strain DH5α was used for cloning and transformation. *Listeria* strains were grown in brain—heart infusion (BHI) broth (Difco) at 37°C. *E.coli* strains were routinely grown in Luria—Bertani (LB) broth at the same temperature. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml for *E.coli*, erythromycin, 300 μg/ml for *E.coli* and 5 μg/ml for *L.monocytogenes*. Restriction enzymes, avian myeloblastosis virus reverse transcriptase, T7 DNA polymerase and ligase were purchased from Pharmacia (Freiburg, FRG) and used as recommended by the manufacturer. The peroxidase-conjugated anti-rabbit immunoglobulin (DAKO laboratories, Hamburg, FRG) was used at a 1:1000 dilution. All other salts and ancillary agents were purchased from Sigma (Deisenhofen, FRG).

DNA sequence analysis

The DNA of the cloned *L.monocytogenes* DNA was determined from double-stranded plasmid templates by dideoxy chain termination (Sanger *et al.*, 1977). Double-stranded templates were denatured and the sequencing reactions were carried out with T7 DNA polymerase as suggested by the commercial supplier in the product literature. Sequencing reactions were primed from vector and custom-made oligonucleotide primers. [α³²P]dATP (800 Ci/mmol) was purchased from Amersham Büchler. The labelled reaction mixtures were separated by electrophoresis on urea—6% polyacrylamide gels, the gels were dried and the sequence read from X-ray film autoradiograms. The sequence data were analysed using the University of Wisconsin Computer Group software (Devereux *et al.*, 1984).

Construction of pAUL53

The fragment carrying sequences internal to the *act*A gene was derived from plasmid pLM53, harbouring spontaneous deletions of the *act*A gene that appeared to have arisen during the cloning of this gene. This pUC18-based plasmid harboured a 1.1 kb DNA segment stretching from nucleotide positions 354 to 1410 of the *act*A sequence. This was excised as an *Eco*RI—*Sal*I fragment and inserted into the same sites on plasmid pAUL-A, a *L.monocytogenes*—*E.coli* shuttle vector that is temperature sensitive for plasmid replication in the former species (Chakraborty *et al.*, 1992).

Recombinants were identified as Lac⁻ colonies and plasmid DNA was analysed using restriction endonucleases to identify plasmids carrying the correct insert. The resulting plasmid, pAUL53, was transformed into *L.monocytogenes* EGD (Chakraborty *et al.*, 1992). Integration of the plasmid was achieved by growing transformed strains at 42°C, a temperature restrictive for the growth of pAUL53.

Southern hybridization

Bacterial chromosomal DNA was isolated as described previously (Leimeister-Wächter and Chakraborty, 1989). Total genomic DNA was cleaved with various restriction endonucleases and electrophoresed overnight at 30 V on 0.7% agarose gels. The DNA was transferred to nitrocellulose sheets (Southern, 1975). The DNA probe for the *act*A gene was amplified using two oligonucleotides flanking the gene (see Figure 1) and was labelled with [α³²P]dATP by the random priming technique (Feinberg and Vogelstein, 1983). Hybridization conditions for the detection of the *act*A gene were as described previously (Leimeister-Wächter and Chakraborty, 1989).

Polyacrylamide gel electrophoresis and immunoblotting

Bacteria were grown overnight at 37°C in BHI broth and supplemented with 5 μg/ml erythromycin where required. SDS—PAGE was performed with a 10% separating gel, using 10 μl of trichloroacetic acid-concentrated supernatants (1 ml) of *Listeria* strains in sample buffer. Polypeptides were visualized by staining the gel with Coomassie Blue R-250.

For immunoblot reactions, proteins were transferred to nitrocellulose paper, reacted with diluted rabbit antiserum at a 1:100 dilution and stained with horseradish peroxidase-conjugated secondary antibody as described previously (Leimeister-Wächter and Chakraborty, 1989).

Peptide synthesis and antiserum production

Peptides corresponding to amino acid residues 41—54, 91—103 and 249—264 were synthesized with an N-terminal cysteine to allow cross-linking to ovalbumin using sulpho-MBS (Pierce-Chemicals) following the procedure recommended by the manufacturer. Antisera were raised in rabbits using standard protocols. 5 mg MBS-activated ovalbumin were reacted with 5 mg reduced peptide overnight at room temperature. Two rabbits were immunized with complete Freund's adjuvant using one-fifth of the MBS—ovalbumin peptide mixture for each rabbit. Two boosters were done with incomplete Freund's adjuvant at three week intervals using half of the original dose.

Plaque formation assay and quantification of bacterial intracellular growth

L929 cells grown as monolayers in 6-well tissue culture dishes (Greiner) were infected with various amounts of bacteria from a culture in the exponential growth phase for 2 h at 37°C in 5% CO₂. Following extensive washing with PBS, the monolayers were overlaid with 0.8% agarose consisting of RPMI 1640, with 500 μg of glucose/ml, 5% fetal calf serum and 50 μg/ml of gentamycin. Plates were incubated for 3—5 days at 37°C in 5% CO₂ and examined daily for areas exhibiting cytopathic effects which are indicative of the intercellular spread of invasive organisms.

For the quantification of intracellular growth, cell monolayers were infected as described above, washed extensively with PBS and incubated with medium containing 10 μg/ml of gentamycin. At the times indicated cells were resuspended in trypsin (0.25% in PBS), lysed by brief sonication and quantitative counts of surviving (i.e. intracellular) bacteria were performed on BHI plates supplemented with 5 μg/ml of erythromycin.

Fluorescence microscopy

Henle cells (Intestine 407) were maintained in DMEM supplemented with 10% fetal calf serum (FCS) in the absence of antibiotics. For infection experiments, cells were grown on coverslips in 6-well tissue culture dishes and placed in serum-free medium 2 h prior to infection. 5 μl of an exponentially growing culture (OD₆₀₀ = 0.5) was added directly to the tissue culture dishes. One hour later, the coverslips were rinsed three times in prewarmed DMEM—FCS medium containing 5 μg/ml gentamycin and incubated for a further 4 h in the same medium. Cells were then rinsed three times in PBS and fixed in formaldehyde (3.7% solution diluted in PBS) for 20 min and finally permeabilized with Triton X-100 (0.2% in PBS) for a further 2 min. Coverslips were processed for double immunofluorescence microscopy using rhodamine-labelled phalloidin and rabbit antiserum to formalinized *L.monocytogenes* EGD, followed by fluorescein-labelled goat anti-rabbit antibodies.

Electron microscopic studies

Infection of Henle cells with bacteria was as described above. Cells were fixed with 0.5% formaldehyde and 0.2% glutaraldehyde directly in the Petri

dishes for 1 h on ice, scraped off the dishes, embedded in 1.5% cacodylate agar and subsequently washed with cacodylate buffer (0.1 M cacodylate, 0.09 M sucrose, 0.01 M $MgCl_2$, 0.005 M $CaCl_2$, pH 7.0). Dehydration of the samples was achieved with a graded series of ethanol as described (Roth *et al.*, 1981). During the dehydration steps the samples were treated with 0.5% uranyl acetate in 30% ethanol at $-20°C$ for 1 h. Infiltration with K4M resin (Chemische Werke Lowi, Waldkraiburg, FRG) was as follows: (i) one part ethanol:one part K4M resin overnight, (ii) one part ethanol:two parts K4M resin for 12 h and (iii) infiltration with pure resin over 2 days with several changes before curing the resin with UV light (366 nm) at $-35°C$ for 1 day and a further 2 days at room temperature. Ultrathin sections were cut with glass knives and the sections were counterstained with uranyl acetate and lead citrate.

Mouse virulence assay

The mouse bioassay was performed as described (Kaufmann, 1984). Mice were infected intravenuously with $\sim 10^3$ bacteria in 70 mM phosphate buffer (pH 7.2) containing 0.25% NaCl. Bacterial numbers were determined at days 2 and 4. Spleens were homogenized and then 0.1 ml portions of appropriate dilutions were plated on BHI agar plates and counted after overnight incubation at 37°C.

Acknowledgements

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Note added in proof

A publication demonstrating that the ActA protein is required for *L.monocytogenes*-induced actin asssembly has recently been reported [Kocks *et al.* (1992) *Cell*, **68**, 521–531]. The sequence data reported here have been deposited in the EMBL Data Library under the accession number X59723.